

ORIGINAL ARTICLE

Platelet-Activating Factor, PAF Acetylhydrolase, and Severe Anaphylaxis

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ABSTRACT

BACKGROUND

Platelet-activating factor (PAF) is an important mediator of anaphylaxis in animals, and interventions that block PAF prevent fatal anaphylaxis. The roles of PAF and PAF acetylhydrolase, the enzyme that inactivates PAF, in anaphylaxis in humans have not been reported.

METHODS

We measured serum PAF levels and PAF acetylhydrolase activity in 41 patients with anaphylaxis and in 23 control patients. Serum PAF acetylhydrolase activity was also measured in 9 patients with peanut allergy who had fatal anaphylaxis and compared with that in 26 nonallergic pediatric control patients, 49 nonallergic adult control patients, 63 children with mild peanut allergy, 24 patients with nonfatal anaphylaxis, 10 children who died of nonanaphylactic causes, 15 children with life-threatening asthma, and 19 children with non-life-threatening asthma.

RESULTS

Mean ($\pm$ SD) serum PAF levels were significantly higher in patients with anaphylaxis (805 ± 595 pg per milliliter) than in patients in the control groups (127 ± 104 pg per milliliter, $P < 0.001$ after log transformation) and were correlated with the severity of anaphylaxis. The proportion of subjects with elevated PAF levels increased from 4% in the control groups to 20% in the group with grade 1 anaphylaxis, 71% in the group with grade 2 anaphylaxis, and 100% in the group with grade 3 anaphylaxis ($P < 0.001$). There was an inverse correlation between PAF levels and PAF acetylhydrolase activity ($P < 0.001$). The proportion of patients with low PAF acetylhydrolase values increased with the severity of anaphylaxis ($P < 0.001$ for all comparisons). Serum PAF acetylhydrolase activity was significantly lower in patients with fatal peanut anaphylaxis than in control patients (P values < 0.001 for all comparisons).

CONCLUSIONS

Serum PAF levels were directly correlated and serum PAF acetylhydrolase activity was inversely correlated with the severity of anaphylaxis. PAF acetylhydrolase activity was significantly lower in patients with fatal anaphylactic reactions to peanuts than in patients in any of the control groups. Failure of PAF acetylhydrolase to inactivate PAF may contribute to the severity of anaphylaxis.

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ANAPHYLAXIS IS A RAPID, POTENTIALLY fatal, immediate hypersensitivity reaction characterized by laryngeal edema, bronchoconstriction, systemic hypotension, and vascular leakage.¹ Factors that predispose persons to anaphylaxis include age, atopy, asthma, mastocytosis, and activating mutations of mast cells.²⁻⁴ Preformed and newly formed biochemical mediators, including histamine, tryptase, carboxypeptidase A, prostaglandin D₂, leukotrienes, and platelet-activating factor (PAF), are released systemically during the degranulation of mast cells and basophils.⁵

PAF is a proinflammatory phospholipid synthesized and secreted by mast cells, monocytes, and fixed tissue macrophages.⁶ Circulating levels of PAF are, in part, controlled by the activity of PAF acetylhydrolase, which is the enzyme that degrades PAF.^{7,8} Transduction of biologic signals after the binding of PAF to its receptor on platelets, monocytes, macrophages, and neutrophils results in many of the manifestations of anaphylaxis.⁹

PAF-receptor antagonists protect against anaphylaxis in mice, rabbits, and rats.⁶ PAF-receptor knockout mice are protected from fatal anaphylaxis, in contrast to wild-type mice, which have intact PAF receptors.¹⁰ Inactivation of PAF by PAF acetylhydrolase also protects against anaphylaxis. Given that PAF is implicated in the pathogenesis of experimental anaphylaxis in animals, we hypothesized that it might also be involved in anaphylaxis in humans. As a corollary, we reasoned that persons with lower levels of PAF acetylhydrolase would have a diminished ability to inactivate circulating PAF and would therefore have more severe manifestations of anaphylaxis than would persons with higher levels of PAF acetylhydrolase. In this study we examined serum PAF levels and PAF acetylhydrolase activity in relation to the severity of anaphylaxis.

METHODS

STUDY DESIGN

We conducted this study in two parts: one was a prospective investigation of the relationship between serum PAF and PAF acetylhydrolase levels and the severity of anaphylaxis, and the other was a retrospective investigation of the relationship between PAF acetylhydrolase and fatal anaphylaxis. In the retrospective study, nine persons who died

from anaphylaxis were compared with persons in five different control groups.

To evaluate the relationship between PAF, PAF acetylhydrolase, and the severity of anaphylaxis, patients with acute allergic reactions were studied prospectively at the time of presentation to the emergency department of a university teaching hospital. There were 41 patients (26 were female and 15 were male), ranging in age from 15 to 74 years. The allergic reactions were triggered by foods in 22 patients, drugs in 12 patients, and insect stings in 2 patients and were idiopathic in 5 patients. Patients were recruited into the study if they met the case-definition criteria for anaphylaxis, as described by Brown et al.¹¹

The severity of the allergic reactions was evaluated according to a published severity score.¹¹ Patients with grade 1 reactions had acute allergic reactions with cutaneous involvement (urticaria, angioedema, rhinitis, or conjunctivitis) and no other organ system involvement, and those with grade 2 reactions had mild-to-moderate manifestations of anaphylaxis (systolic blood pressure, >90 mm Hg; respiratory rate, <25 breaths per minute; and a normal score on the Glasgow Coma Scale). Those with grade 3 reactions had severe manifestations, with cutaneous, gastrointestinal, and potentially life-threatening respiratory or cardiovascular signs and symptoms (any one of the following: loss of consciousness, dizziness, lightheadedness, systolic blood pressure of <90 mm Hg, or a Glasgow Coma Scale score <15, as well as one or more of the following: dyspnea, wheeze, hoarseness, or bronchospasm, plus one or more of the following: stridor, cyanosis, laryngeal edema, or a respiratory rate ≥25 per minute). Twenty-three healthy volunteers served as controls (16 men and 7 women; mean [±SD] age, 30.8±9.8 years; range, 20 to 51).

The relationship between PAF acetylhydrolase and fatal anaphylaxis was examined retrospectively. Serum samples were collected from three male and six female patients who had fatal anaphylactic reactions to peanuts (mean age, 14.3±4.6 years; range, 9 to 24). These samples, which were collected by three different coroners at the time of the fatal episode, were stored at -20°C and assayed, in duplicate, for PAF acetylhydrolase activity. The samples were coded and assayed in duplicate in a blinded fashion for determination of PAF acetylhydrolase.

In addition, serum samples were obtained from five comparison groups. The first comparison group consisted of 49 nonallergic adults (22 men and 27 women; mean age, 37.9 ± 11.3 years; range, 19 to 63), and the second group consisted of 26 nonallergic children (15 boys and 11 girls; mean age, 9.2 ± 4.0 years; range, 3 to 16). Samples from these two control groups were obtained at the time of routine preoperative blood work for elective procedures. The third group consisted of 63 healthy children (35 boys and 28 girls; mean age, 6.5 ± 3.9 years; range, 2 to 19) with mild peanut allergy. These children had had previous allergic reactions to peanuts that were characterized only by urticaria, angioedema, or both, with positive skin tests to peanut protein that showed wheals 8 mm or more in diameter. The fourth group consisted of 24 patients with nonfatal acute anaphylactic reactions to peanuts; demographic information on these patients is not available. The fifth group consisted of 10 children (8 boys and 2 girls; mean age, 11.5 ± 3.5 years; range, 4 to 15) who died of causes unrelated to anaphylaxis (2 from drowning, 1 from homicide, 4 from crib death, and 3 from motor vehicle accidents). These samples were provided by provincial coroners.

Because asthma is recognized as a risk factor for fatal anaphylaxis,^{3,12} we performed additional analyses to determine PAF acetylhydrolase activity in subjects with life-threatening and non-life-threatening asthma in order to test whether PAF acetylhydrolase level is a risk factor distinct from severe asthma. The group with life-threatening asthma consisted of 15 children (3 to 16 years of age) who were admitted to pediatric intensive care units with acute asthma requiring ventilation or high-dose intravenous bronchodilators. The group with non-life-threatening asthma consisted of 19 children (4 to 17 years of age) who had acute exacerbations of asthma and were treated in hospital outpatient clinics or in primary care offices in the United Kingdom.

The study was approved by the research ethics board at St. Michael's Hospital in Toronto. Informed consent was obtained from all subjects or their parent or guardian.

MEASUREMENT OF PAF LEVELS AND PAF HALF-LIFE

Details of measurements, materials, and suppliers are outlined in the Methods section of the Supplementary Appendix (available with the full text

of this article at www.nejm.org). The concentration of PAF in samples of human blood was measured with the Platelet Activating Factor ³H-Scintillation Proximity Assay (SPA) system (Amersham Biosciences). The half-life of PAF in human serum as a function of PAF acetylhydrolase activity was determined by the method of Stafforini et al.¹³

MEASUREMENT OF PAF ACETYLHYDROLASE ACTIVITY AND STABILITY

The activity of PAF acetylhydrolase was assayed according to the method of Miwa et al.¹⁴ with minor modifications (see the Supplementary Appendix). For patients with fatal peanut anaphylaxis, we considered levels that were 2 SD or less above the mean (i.e., ≤ 20 nmol per milliliter of serum per minute) to be low on the basis of the first four of the nine fatalities. Moreover, 48 adult control patients (98%) and all but 5 of all control patients (93%) had PAF acetylhydrolase activities above 20 nmol per milliliter per minute.

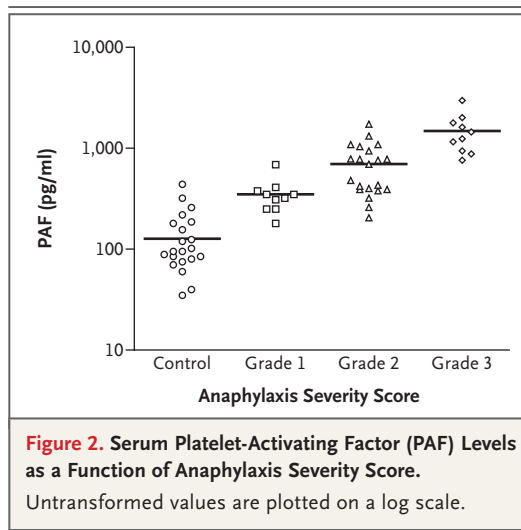
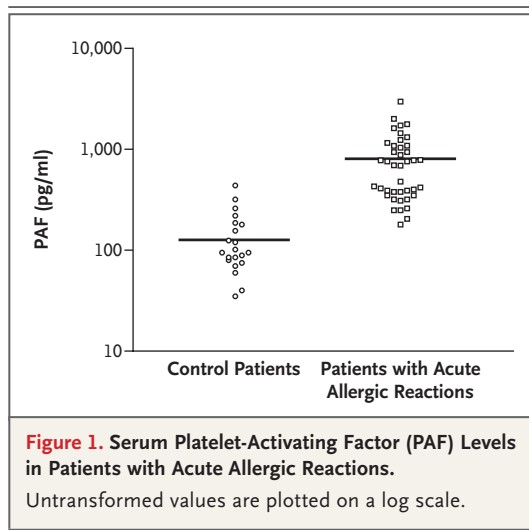
The serum samples may have been stored under differing conditions, subjecting PAF acetylhydrolase to thermal stresses. In order to evaluate the effect of handling and storage conditions on PAF acetylhydrolase activity, the serum samples were subjected to a range of temperatures and freeze-thaw cycles and assayed for residual PAF acetylhydrolase activity, as detailed in the Supplementary Appendix.

EFFECT OF EPINEPHRINE ON PAF ACETYLHYDROLASE ACTIVITY

Epinephrine is used in the treatment and resuscitation of patients with anaphylaxis and may potentially influence serum PAF acetylhydrolase levels. To examine the effect of intramuscular injection of 0.3 mg of epinephrine on PAF acetylhydrolase activity, blood was drawn from eight healthy children with a history of anaphylaxis to food, hymenoptera venom, or other substances, who were participants in a clinical pharmacology study but were in stable health at the time of the study.¹⁵ PAF acetylhydrolase activity was assayed in serial blood samples drawn before and 5, 10, 15, 20, 30, 40, 60, 90, 120, and 180 minutes after intramuscular epinephrine injection.

STATISTICAL ANALYSIS

The PAF data were log transformed (1 was added to the one zero-level sample in the control group),



and the PAF acetylhydrolase data were not. The levels of PAF and PAF acetylhydrolase in patients with different grades of anaphylaxis were compared by analysis of variance for continuous analysis and by the chi-square test for categorical analysis. The analysis was performed for all 41 patients as a group and separately for the 22 patients with reactions triggered by food. Levels of PAF acetylhydrolase were examined both as continuous variables and as categorical variables (with the use of a cutoff point of 20 nmol per milliliter per minute). We compared continuous variables using t-tests and categorical variables using chi-square or two-tailed Fisher's exact tests as appropriate; all analyses were conducted with SAS software, versions 6.12 and 8.2. We compared each of the five control groups with the group of nine patients who died of peanut anaphylaxis, and we adjusted for multiple comparisons by considering P values less than 0.01 to indicate statistical significance.

RESULTS

PAF ACETYLHYDROLASE ACTIVITY AND THE HALF-LIFE OF PAF IN SERUM

The half-life of exogenous PAF was measured as a function of PAF acetylhydrolase activity. Three serum samples with PAF acetylhydrolase activities of 7.8, 24.8, and 47.6 nmol per milliliter per minute were used, representing low, intermediate, and high levels, respectively, of PAF acetylhydrolase activity. The half-life of exogenous PAF was 13.6 ± 0.9 minutes in serum with the lowest PAF acetylhy-

drolase activity, 6.0 ± 0.8 minutes in serum with intermediate PAF acetylhydrolase activity, and only 3.8 ± 0.4 minutes in serum with the highest PAF acetylhydrolase activity (Fig. 1 in the Supplementary Appendix).

SERUM PAF LEVELS IN PATIENTS WITH ACUTE ALLERGIC REACTIONS

PAF was measured in the blood of 41 patients presenting to an emergency department with acute allergic reactions. For the continuous analysis, the mean serum PAF levels among patients with acute allergic reactions were significantly greater than those among patients in the control groups (805 ± 595 pg per milliliter [6.5 ± 0.7 pg per milliliter after natural-log transformation] vs. 127 ± 104 pg per milliliter [4.3 ± 1.5 pg per milliliter after natural-log transformation], $P < 0.001$ for log-transformed data) (Fig. 1). The relationship between serum PAF concentrations and the severity of the allergic reactions is shown in Figure 2. Pairwise comparison showed that for the log-transformed values, the PAF levels in control patients were significantly different from those in patients with grade 1, 2, or 3 anaphylaxis ($P < 0.001$ for all comparisons). There were significant differences among the four groups in PAF levels according to analysis of variance ($P < 0.001$ for log-transformed data, $R^2 = 0.57$). Analysis of variance also showed significant differences in PAF levels among patients with one of the three grades of allergic reactions ($P < 0.001$). Pairwise comparisons also showed that for the log-transformed data, patients with one of

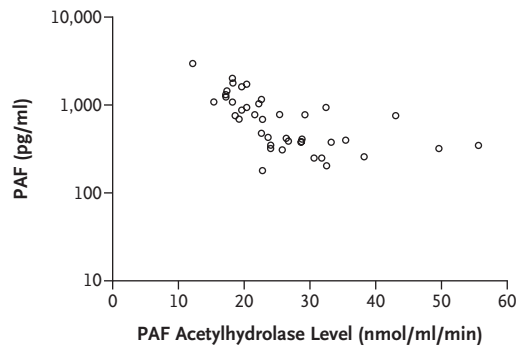


Figure 3. Serum Platelet-Activating Factor (PAF) Levels as a Function of PAF Acetylhydrolase Activity in Patients with Acute Allergic Reactions.

Untransformed values are plotted on a log scale.

the three grades of allergic reaction had PAF levels that were significantly different from those of patients with one or the other of the other two grades ($P < 0.01$).

For the categorical analysis, on the basis of a cutoff of 400 pg per milliliter as the upper limit of normal, PAF levels were elevated in 26 of the 41 patients with allergic reactions (63%), but in only 1 of the 23 control patients (4%, $P < 0.001$ by the chi-square test with 1 df). The proportion of patients with elevated PAF values increased monotonically across groups, from 1 patient (4%) in the control group, to 2 (20%) in the group with grade 1 allergic reactions, to 15 (71%) in the group with grade 2 reactions, and to all 10 (100%) in the group with grade 3 reactions ($P < 0.001$ with 3 df). Our results are in agreement with previously reported levels of PAF in nonallergic persons measured by the same methods.^{16,17}

RELATIONSHIP OF PAF AND PAF ACETYLHYDROLASE

PAF acetylhydrolase is the major enzyme that hydrolyzes PAF to the biologically inactive form, lyso-PAF. There was a significant inverse correlation between PAF levels and PAF acetylhydrolase activity in the 41 patients with acute allergic reactions (Fig. 3); the correlation was stronger in the non-parametric analysis (Spearman rank-correlation coefficient, -0.75 ; $P < 0.001$).

ANALYSIS OF PAF ACETYLHYDROLASE LEVELS ACCORDING TO GRADE OF ANAPHYLAXIS

All Patients

For the continuous analysis, there was a trend in the mean levels of PAF acetylhydrolase activity; the

levels were highest in controls and lowest in patients with grade 3 anaphylaxis, although the differences were not significant (Table 1). For the categorical analysis, the proportion of patients with low PAF acetylhydrolase values increased with an increase in the severity of anaphylaxis (Table 1); the proportion was highest among patients with grade 3 anaphylaxis, and the difference in proportions among the groups was significant ($P < 0.001$).

Patients with Allergic Reactions to Foods

For the continuous analysis, there was a trend in the mean levels of PAF acetylhydrolase activity; the levels were highest in control patients and lowest in patients with grade 3 anaphylaxis caused by foods, but the differences were not significant (Table 1B in the Supplementary Appendix). For the categorical analysis, the proportion of patients with low levels of PAF acetylhydrolase activity increased with increasing severity of food-induced anaphylaxis (Table 1B in the Supplementary Appendix); despite the small numbers of patients, the difference in proportions among the groups was significant ($P = 0.007$).

PAF ACETYLHYDROLASE ACTIVITY IN PEANUT-ALLERGIC PATIENTS AND CONTROL PATIENTS

The relationship between PAF acetylhydrolase and fatal anaphylaxis was studied retrospectively in peanut-allergic patients. The mean serum PAF acetylhydrolase level in patients who had fatal anaphylactic reactions to peanuts (14.5 ± 3.4 nmol per milliliter per minute) was significantly lower than the levels in nonallergic adult control patients (34.9 ± 10.6 nmol per milliliter per minute), non-allergic pediatric control patients (27.7 ± 8.5 nmol per milliliter per minute), and children with peanut-induced urticaria and angioedema (25.2 ± 5.7 nmol per milliliter per minute, $P < 0.001$ for all comparisons). There was no significant difference in mean serum PAF acetylhydrolase activity among these three control groups (Table 2 and Fig. 4).

The mean PAF acetylhydrolase level was significantly lower among patients who had fatal anaphylactic reactions to peanuts than among nonallergic adults or children (Table 2). All 9 patients who died had low PAF acetylhydrolase values (≤ 20 nmol per milliliter per minute), as compared with only 1 of 49 nonallergic adults (2%, $P = 0.001$) and 4 of 26 nonallergic children (15%, $P < 0.001$ by two-tailed Fisher's exact test).

Table 1. Analysis of PAF Acetylhydrolase Activity According to Grade of Anaphylaxis.*

Variable	Control (N=23)	Grade 1 (N=10)	Grade 2 (N=21)	Grade 3 (N=10)	P Value
PAF acetylhydrolase activity — nmol/ml/min	30.7±8.2	29.5±9.7	25.9±8.3	22.1±9.0	0.13†
PAF acetylhydrolase activity ≤20 nmol/ml/min — no. (%)	0	0	5 (24)	7 (70)	<0.001‡

* PAF denotes platelet-activating factor. Plus-minus values are means ±SD.

† R² was 0.11.

‡ The P value was calculated with 3 df.

Table 2. Comparison of PAF Acetylhydrolase Activity in Patients with Fatal Peanut Anaphylaxis and Control Groups.*

Variable	Fatal Peanut Anaphylaxis (N=9)	Adult Control (N=49)	Pediatric Control (N=26)	Children with Peanut Allergy (N=63)	Nonfatal Peanut Anaphylaxis (N=24)	Nonanaphylactic Deaths (N=10)
PAF acetylhydrolase activity — nmol/ml/min						
Mean	14.5±3.4	34.9±10.6†	27.7±8.5†	25.2±5.7†	29.7±9.1†	26.4±7.2†
Range	9.7–18.6	19.0–59.8	11.4–48.2	14.2–41.0	12.8–45.8	15.2–34.6
PAF acetylhydrolase activity ≤20 nmol/ml/min — no. (%)	9 (100)	1 (2)†	4 (15)†	13 (21)†	5 (21)†	3 (30)‡

* PAF denotes platelet-activating factor. Plus-minus values are means ±SD.

† P<0.001 for the comparison with the fatal-peanut-anaphylaxis group.

‡ P<0.01 for the comparison with the fatal-peanut-anaphylaxis group.

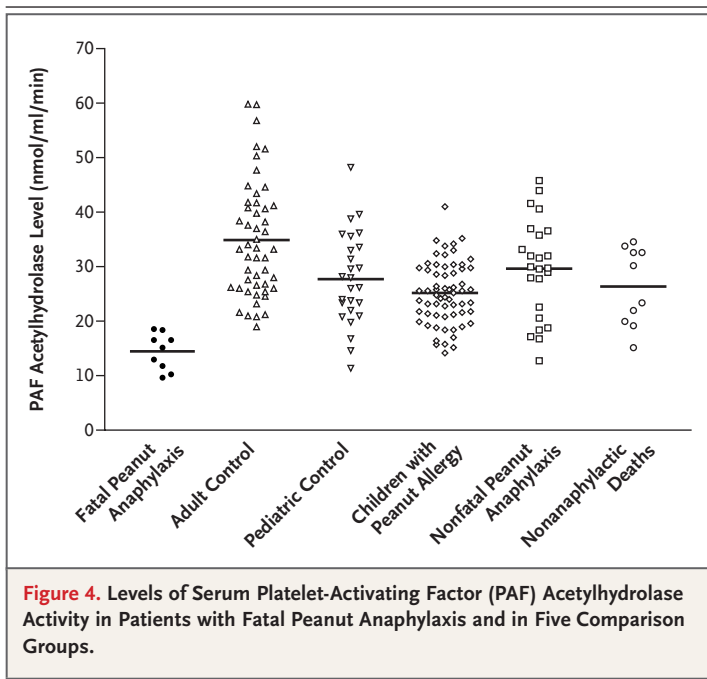
There were significant differences in mean PAF acetylhydrolase levels between the 9 patients who died of anaphylactic reactions to peanuts and the 63 children with peanut-induced urticaria and angioedema (P<0.001) (Table 2). The mean PAF acetylhydrolase level in mildly peanut-allergic subjects (25.2±5.7 nmol per milliliter per minute) was almost identical to those of children who died of nonanaphylactic causes and of nonallergic children (26.4±7.2 and 27.7±8.5 nmol per milliliter per minute, respectively). All 9 of those who died of anaphylactic reactions to peanuts had low PAF acetylhydrolase levels (≤20 nmol per milliliter per minute), whereas only 13 of the 63 children with mild peanut allergy (21%) had low PAF acetylhydrolase levels (P<0.001 by two-tailed Fisher's exact test). The group classification (fatal vs. mild allergic reactions to peanuts) explained 35% of the variation in PAF acetylhydrolase levels.

The mean PAF acetylhydrolase level was significantly lower among patients who had fatal anaphylactic reactions to peanuts than among those who had nonfatal reactions (P<0.001) (Table 2). Only five patients in the nonfatal group (21%) had low PAF acetylhydrolase levels, as compared with all nine of those who died (P<0.001).

The mean PAF acetylhydrolase level among the 9 patients who died of anaphylactic reactions to peanuts was significantly lower than that among the 10 children who died of nonanaphylactic causes (P<0.001) (Table 2). All nine patients who died of anaphylaxis but only three who died of other causes (30%) had low levels of PAF acetylhydrolase (P<0.01 by two-tailed Fisher's exact test).

To assess the specificity of the findings in fatal anaphylaxis, separate analyses were conducted in which PAF acetylhydrolase levels were measured in patients with life-threatening asthma and in those with non-life-threatening asthma. There was no significant difference between these two groups (28.6±7.0 and 26.7±7.9 nmol per milliliter per minute, respectively). These levels were almost identical to those of nonallergic children (27.7±8.5 nmol per milliliter per minute) and significantly greater than those of patients with fatal peanut anaphylaxis.

The results of studies of the stability of PAF acetylhydrolase, the effect of epinephrine on PAF acetylhydrolase activity, and the effect of glucocorticoids on PAF acetylhydrolase activity are shown in the Supplementary Appendix. PAF acetylhydrolase activity was not influenced by



the administration of either epinephrine or glucocorticoids.

DISCUSSION

We have shown that circulating PAF levels are increased and circulating PAF acetylhydrolase activity is decreased in proportion to the severity of organ system involvement in patients with acute allergic reactions triggered by foods, medications, or insect stings. Our retrospective analysis of patients with peanut allergy showed that PAF acetylhydrolase activity was significantly lower in patients with fatal peanut anaphylaxis than in those with mild allergic reactions to peanuts and persons in other control groups. These data are consistent with a role for PAF in the pathobiology of acute allergic reactions.

PAF is a highly potent biologic mediator, active at concentrations as low as 10^{-12} M.¹⁸ Because of its rapid inactivation by PAF acetylhydrolase, it has a short elimination half-life, ranging from 3 to 13 minutes. Patients with anaphylaxis often present to the emergency department minutes or even hours after their symptoms begin, and for the purposes of clinical investigation, it takes additional time to obtain informed consent, enroll patients in the study, and perform venipuncture. Consequently, our results probably represent a

conservative underestimate of the peak PAF concentrations generated during acute allergic reactions. Even so, the difference between PAF levels in patients with anaphylaxis and nonallergic persons was highly significant. Mean PAF levels in patients with grade 1 anaphylaxis, in those with grade 2 anaphylaxis, and in those with grade 3 anaphylaxis were approximately 2.5 times, 5 times, and more than 10 times greater, respectively, than the levels in the control patients.

The biologic half-life of PAF is determined primarily by the rate of inactivation of PAF by PAF acetylhydrolase.^{8,18} Hence, the slower the inactivation of PAF, the more severe the manifestations of anaphylaxis may be. Conversely, more rapid rates of inactivation of PAF might be expected to result in milder allergic reactions. Indeed, we have found a significant inverse correlation between PAF acetylhydrolase activity and PAF levels as well as an inverse relationship between the severity of allergic reactions and PAF acetylhydrolase activity. This relationship was noted in the group analysis of all patients with anaphylaxis as well as in the subgroup of patients with allergic reactions triggered by foods.

There is compelling evidence from studies in animals that links PAF to experimental anaphylaxis. We have found that PAF levels correlate with the severity of anaphylaxis¹⁹ and may be an important determinant of outcome. This is not to say that PAF acts in isolation; there are undoubtedly important interactions with other mediators generated during anaphylaxis, as well as host factors, such as coexistent cardiovascular disease or asthma. Given the findings of normal PAF acetylhydrolase levels in persons with life-threatening asthma and in those with non-life-threatening asthma, we suggest that PAF acetylhydrolase deficiency is an independent risk factor for fatal anaphylaxis, separate from asthma. However, PAF acetylhydrolase deficiency alone is not sufficient to predispose to fatal anaphylaxis; a person must be sensitized to the allergen, and other clinical factors, such as age and presence or absence of asthma, are also important determinants of outcome.

These data provide the rationale for the development of drugs to selectively block the actions of PAF, both as rescue therapy in cases of acute anaphylaxis and potentially as long-term preventive treatment for those at highest risk for fatal anaphylaxis. Demonstration of the efficacy of such

agents would serve to ensure that PAF is a link in the chain of causality between allergic events and the anaphylactic phenotype. Although these data show that elevated levels of PAF and decreased PAF acetylhydrolase activity correlate with anaphylaxis, further studies will be required to assess the usefulness of PAF acetylhydrolase as a biochemical marker to identify patients at high-risk for fatal anaphylaxis, so that appropriate risk-reduction strategies²⁰ can be implemented.

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Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Vadas P, Gold M, Perelman B, et al. Platelet-activating factor, PAF acetylhydrolase, and severe anaphylaxis. N Engl J Med 2008;358:28-35.

Supplementary Appendix

METHODS

Materials

Radiolabeled PAF (1-O-hexadecyl-[2acetyl- ^3H]-sn-glycero-3-phosphocholine, 499.5 Gbq/mmol) was purchased from NEN Life Science Products (Boston, MA). Unlabeled PAF and lyso-PAF, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine, egg yolk phosphatidylcholine, pefabloc (4-[2-aminoethyl]benzenesulfonyl fluoride) were obtained from Sigma (Sigma-Aldrich Canada, Oakville, Canada). Bond-Elute C18 columns were from Varian Inc. Platelet activating factor ^3H scintillation proximity assay (SPA) system and liquid scintillation fluid (ACS) were supplied by Amersham Biosciences (Oakville, Canada). Pre-Coated TLC Plates SILICA GEL 60 (layer thickness 0.25 mm, 20 x 20 cm) were purchased from Merck (Damstadt, Germany). All other chemicals were from Sigma.

Measurement of Platelet Activating Factor in Human Blood

Blood samples to be assayed for PAF were treated as recommended by NEN Life Sciences (Boston, MA). One volume of blood was vigorously shaken with an equal volume of 20% acetic acid to inactivate PAF acetylhydrolase. Two volumes of 10% acetic acid were added and the suspension was shaken again. After centrifugation (12 000g, 60 min) the supernatant was recovered and the

pellet was subjected to a second extraction with 10% acetic acid in a similar manner. These extracts were combined.

PAF was initially purified by chromatography on C-18 Octadecyl mini-columns (200 mg of sorbent), according to the manufacturer's instructions. Further purification of the extracted PAF was performed by the method of Bligh and Dyer with a mixture of chloroform-methanol-water (1:1:0.9) (1). The lower chloroform phase, containing PAF, was removed and evaporated to dryness under a gentle stream of nitrogen. Before assay, PAF was reconstituted with assay buffer by vortexing for 15 min.

The concentration of PAF in samples was measured using the Platelet Activating Factor ^3H -Scintillation Proximity Assay (SPA) system (Amersham Biosciences, Oakville, Canada). PAF was measured in the range of 0.2 - 12.8 ng/ml. Assay specificity and sensitivity were determined by addition of known amounts of PAF and subjecting each concentration to the extraction and purification procedures, followed by SPA. The assay was sensitive to 20-25 pg/tube and was reproducible from assay to assay. The inter- and intra-assay coefficients of variability were <15%. The amount of metabolite measured was corrected for recovery and expressed as pg per ml of blood.

Half-Life of PAF in Human Serum

The half-life of authentic PAF in human serum as a function of PAF-AH activity, was determined by the method of Stafforini et al (2). Reaction mixtures

containing serum with different PAF-AH activities were incubated with 50 nmol/l (5×10^{-8} M) [2-acetyl- ^3H]-PAF (499.5 Bq [2.99×10 dpm]/pmol]) at 37°C for various periods of time. Aliquots (150 μl) were removed at the stated times directly into an equal volume of 20% acetic acid (for inactivation of PAF-AH in serum) and the total lipids were then extracted from the samples by the method of Bligh and Dyer (1). All experiments were repeated in triplicate.

First, the samples were mixed with chloroform /methanol/ water 1:2:0.8 (by vol). The solvent mixture was phased by adding the appropriate volumes of chloroform and water to a final ratio of 1:1:0.9. The mixture was shaken vigorously and the phases were separated by centrifugation. The lower chloroform phase was removed and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl of chloroform and applied to prepared silica gel G plates. Prior to use, Silica gel G plates were placed in a tank containing a neutral solvent system of chloroform / methanol / water (65:35:6, v/v/v). The solvent was allowed to migrate to the top and the plate was removed and air-dried. It was then activated by heating to 110°C for 30 min. The activated plates were developed in a solvent system of chloroform / methanol / water (65/35/6) as previously described (2), using authentic PAF and lyso-PAF as standards. Lipids were located by iodine staining and the appropriate zone, co-migrating with PAF standard, was scraped and radioactivity measured in a liquid scintillation counter.

Recovery rate

To evaluate the efficiency of recovery of PAF as well as the reproducibility of the technique, the same amount of ^3H -PAF that was added to serum was applied directly to the thin layer plate without extraction. The efficiency of recovery, $84 \pm 8\%$, was found to be very consistent and satisfactory in 13 independent experiments.

Measurement of serum PAF acetylhydrolase activity

The activity of PAF acetylhydrolase was assayed according to the method of Miwa et al (3) with minor modifications. [2-acetyl- ^3H]-PAF (1-hexadecyl-2-[^3H]acetyl-sn-glycero-3-phosphocholine and 400 nm of non-radiolabeled PAF were mixed to a specific radioactivity of $12.5 \mu\text{Ci}/\mu\text{mol}$. After evaporation of the solvent under a stream of nitrogen, 1 ml of 0.1 M HEPES buffer (pH 7.2) containing 1 mM fatty acid-free bovine serum albumin was added, and the solution was sonicated for 5 min (30 sec increments) at 4°C (Vibra Cell Sonicator, Sonifier, Model W350, Branson Sonic Corp., Danbury, USA). The solution was stored frozen for not more than 1 week to avoid non-enzymatic hydrolysis. The sonication step was repeated each time the substrate was thawed. The working solutions were $160 \mu\text{M}$ with 9000-11000 cpm/nmol.

Results were expressed as rate of PAF hydrolysis (amount of released [^3H]-acetate in nmol per ml serum per minute at 37°C). The recovery of released [^3H]-acetate, as estimated by extraction of PAF samples, which had been completely hydrolysed with 1 M NaOH at 50°C for 30 min, was greater than 96%.

Each assay mixture contained 5 μ l of human serum and 10 μ l of substrate ([2-acetyl- ^{3} H]-PAF), and the final volume was adjusted to 25 μ l with 0.1 M HEPES-BSA buffer. The final concentration of the substrate was 160 μ M. The assay mixture was incubated for 10 min at 37°C. The reaction was stopped in an ice bath. The unreacted [2-acetyl- ^{3} H]-PAF was immediately bound to an excess of BSA (16.7 mg/ml final concentration) and precipitated by addition of trichloroacetic acid (final concentration 8% v/v) for 10 min at 0°C. Denatured protein was removed by centrifugation at 5000 g for 15 min. The released [^{3} H] acetate was measured by scintillation counting in 6 ml of ACS scintillation fluid.

Before calculating enzyme activities, blanks were subtracted. Blanks were prepared using acid treated serum (pH 2.0, 30 min, 37° C) to inactivate PAF acetylhydrolase activity.

Stability of PAF-AH

Serum samples from individuals with fatal peanut anaphylaxis and from various control groups may have been stored under differing conditions, subjecting PAF-AH to thermal stresses. In order to evaluate the effect of handling and storage conditions on PAF-AH activity, sera were stored at different temperatures. The stability of PAF-AH stored at 4 $^{\circ}$ C and 23 $^{\circ}$ C and subjected to repeated cycles of freeze- thawing was assessed. PAF-AH levels were measured at specified times

following storage at 23⁰C (2, 4, 8, 24 and 48 hours) and at 4⁰C (5 and 10 days) and following 5 sequential cycles of freezing and thawing. To assess the stability of PAF-AH to long term storage, serum samples stored at -70⁰C since 1998 were re-assayed for PAF-AH and compared to results of assay results prior to storage.

RESULTS

Stability of PAF-AH: To assess the stability of PAF-AH to long term storage at -70⁰C, PAF-AH activity was measured in sera (n=25) in 1998 and again in 2006. Mean PAF-AH activity in fresh sera in 1998 was 27.04 ± 9.92 vs 22.98 ± 7.23 nmol/ml/min in the same serum samples eight years later (p=0.1047). PAF-AH activity was measured in 6 fresh sera subjected to 5 freeze-thaw cycles. Residual activity ranged from 90 – 105% of that in fresh sera. Sera (n=6) stored at 4⁰C for 10 days retained 96 – 102% of initial activity, and sera stored at 23⁰C for up to 48 hours retained 84 – 92% of PAF-AH activity of fresh sera.

Effect of Epinephrine on PAF-AH Activity: The effect of a typical intramuscular dose of epinephrine on serum PAF-AH activity was assessed. The pre-injection baseline plasma epinephrine concentration was 339 ± 115 pg/ml. A maximum plasma epinephrine concentration of 2136 ± 351 pg/ml was achieved by 8±2 min. After 40 min, epinephrine concentration was in the range of 400-500 pg/ml, until 180 min. Mean PAF-AH activity prior to injection of epinephrine was 25.4 nmol/min/ml. PAF-AH activity did not change significantly over the course of 3

hours. Serial PAF-AH activity ranged from 28.5 ± 6.6 (SD) to 30.8 ± 6.9 nmol/min/ml over the course of 180 min. following epinephrine injection.

Effect of glucocorticosteroids on PAF-AH Activity: Patients with both LTA and NLTA were treated with glucocorticosteroids, expressed as micrograms of beclomethasone equivalent. The large standard deviation relative to the mean of steroid dosage (mean $\pm$ SD, 537 ± 466) suggested the variable was skewed or was not normally distributed; the lognormal distribution had identical mean and SD of 5.998 ± 0.758 with median identical to the mean (5.991). The dosages of steroids were compared between the children with LTA and with NLTA with the t-test. There was no significant difference in steroid dosage between LTA (mean 644 ± 467) and NLTA (mean 473 ± 470), whether untransformed ($p=0.40$) or log-transformed ($p=0.29$). The asthma type explained very little of the variation (R-square 3.2% and 5.1% respectively).

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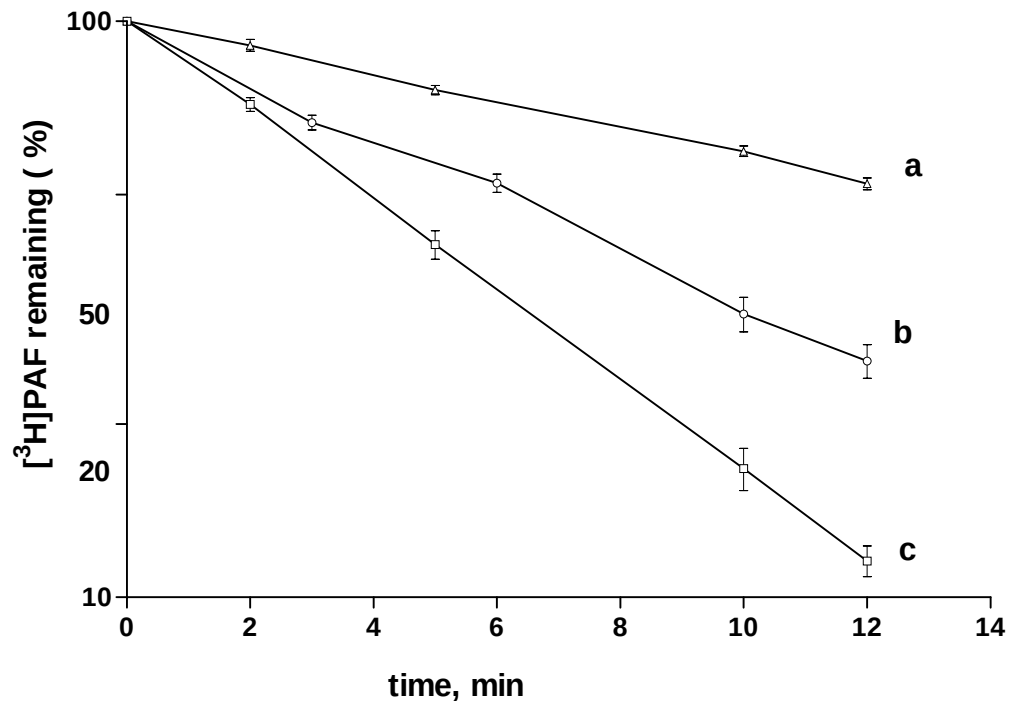


Figure Legends

Supplemental Figure 1. Half-life of exogenous PAF as a function of PAF-AH activity. a. serum with PAF-AH activity of 7.8 nmol/ml/min; b. serum with PAF-AH activity of 24.8 nmol/ml/min; c. serum with PAF-AH activity of 47.6 nmol/ml/min.